was taken for protein concentration testing by the Bio-Rad method (Bio-Rad Laboratories, Hercules, CA) and for SDS-PAGE.

[59] The bulk clarified promastigotes lysate was tightly sealed and stored at 4 ± 2 °C until treated with heat. 25% glycerol was added to the bulk clarified promastigotes lysate to give a final concentration of 1% and then mixed by swirling. The total weight of the in process bulk was determined. Then the bulk clarified promastigotes lysate was incubated for 30 ± 2 minutes using a water bath at 93 ± 2 °C. After heat treatment, the bulk was cooled at 4 ± 2 °C. Aseptically two 0.5 ml aliquots were taken and retained at -80 ± 10 °C. One 1.0 ml sample was taken for protein concentration testing by the Bio-Rad method (Bio-Rad Laboratories, Hercules, CA) and for SDS-PAGE.

[60] The final clarified lysate protein concentration was aseptically adjusted to 0.35 ± 0.05 mg/ml with 0.4% phenol buffer. Then samples were aseptically obtained and assayed for protein content by BCA, endotoxin content LAL (gel-clot), pH, sterility, HPLC, purity by SDS-PAGE, color and appearance.

[61] An Omnispense dispensing pump (Wheaton Science Products, Millvilee, NJ) was used to dispense 1.0 g ± 5% of the final clarified lysate into 10 ml depyrogenized, sterile, glass vials. The bulk final clarified lysate was swirled on an orbital shaker at 60 rpm during the filling operation to ensure homogeneity. The final vials were inspected and weight checked. The vials were labeled and tested for sterility, pH, protein concentration, LAL, SDS-PAGE, HPLC and immunogenicity. Figure 1 is a flow diagram of process for making the Leishmaniasis microfluidized lysate of the present invention.

Example 3

Skin Test Antigen Assay in 10 Subjects

[62] A microfluidized lysate preparation of *L. tropica* prepared according the Example 2 was tested in 10 *Leishmania* naïve human subjects. The microfluidized lysate preparation further included Tween-80® and dextran. The subjects received four doses two weeks apart of 0.1 ml of the microfluidized lysate preparation in escalating concentrations. The first dose had 0.25 μg, the second dose had 2.5 μg, the third dose had 8.0 μg, and the fourth dose had 25.0 μg of total protein. 25.0 μg of the preparation was found to be safe in six of the 10 subjects. One subject developed clear rhinorrhea and nasal pruritus within minutes of receiving the first dose. Four hours later, transient urticaria at the test and control-diluent sites occurred. This reaction was consistent with

systemic manifestations of type I hypersensitivity to the dextran and diluent control. Three additional subjects also withdrew from the study after developing induration at the site of administration. The remaining subjects, except one subject who withdrew from the study due to a move out of the area, received three additional 25.0 µg doses of the microfluidized lysate to evaluate sensitization. The subject who moved from the area received only two additional 25.0 µg doses without difficulty.

[63] As it was believed that the dextran caused the hypersensitivity in the subjects, the microfluidized lysate was reformulated. The reformulated microfluidized lysate preparation of *L. tropica* comprising 0.4% Phenol as a preservative was tested in 15 *Leishmania* naïve human subjects and was found to be safe as a single injection at doses of 0.38 µg, 3.8 µg, and 38.0 µg.

Example 4

Heat-Treated Leishmania Skin Test Injectable Study

[64] 60 active leishmania subjects, 60 healthy leishmania subjects, and 60 healed leishmania subjects ages 18-55 are recruited to evaluate the safety of the microfluidized lysate preparation, determine the specificity and sensitivity of varying antigen doses, evaluate the sensitizing capacity of the microfluidized lysate preparation, and to compare the intensity of the induration responses evoked (cross-reactivity) when heterologous and homologous antigens are used.

[65] A subject is deemed to be clinically diagnosed with an active Leishmaniasis infection upon the demonstration of motile promastigotes in aspirate cultures or microscopic Leishmania amastigotes in samples obtained from lesions obtained from the subject. The parasite may be visualized by conventional methods. A subject is deemed to be clinically healed upon 100% reepitheliazation of the ulcer.

[66] All subjects are tested with an anergy panel comprising Mumps Skin Test Antigen, 40 CFU/ml, MSTA®, Connaught Labs Inc. (Swiftwater, PM), and Candida albicans skin test antigen, Candin®, Allermed Laboratories, Inc. (San Diego, CA), before receiving the preparations. Subjects who have a positive response to at least one of the antigens of the anergy panel, an induration of greater than about 5 mm, may participate.

[67] Subjects who have a history of atopy, allergic reactions, or asthma will be excluded from the study including those who are allergic to phenol, pharmaceutical detergents or glycol. Also excluded are those subjects taking steroids, antihistamines,

cimetidine, and immunosupressants, as well as those who had a splenectomy or have an active medical or psychiatric conditions that may increase the risks associated with participation in the study or interfere with the interpretation of study results. Pregnant or nursing subjects will be excluded. Subjects having active cutaneous Leishmaniasis with scars, i.e. possible re-infections and residivant Leishmaniasis will be excluded. Subjects having been immunized within 4 weeks prior to the start of the study will be excluded as well as those having anergy on delayed type hypersensitivity testing (less than about 5 mm of induration).

[68] Primary endpoints are the occurrence of local or systemic reactions to the skin or the occurrence of non-specific immune responses to the skin tests. The microfluidized lysate preparations are considered safe if there are no clinically significant local or systemic reactions in the healthy and healed subjects. The microfluizided lysate preparations will also be considered safe if no severe reactions are determined in active leishmania subjects. Secondary endpoints are the size of the induration which accompanies the delayed type hypersensitivity response (potency); the percentage of subjects with Leishmaniasis in which the preparation induced a positive response about 5 mm or greater delayed type hypersensitivity reaction (sensitivity), the percent of healthy subjects in which the preparation fails to induce a positive delayed type hypersensitivity reaction (specificity).

[69] The microfluidized lysate preparations are first tested in healthy subjects, followed by healed subjects, and followed by visceral or cutaneous subjects. In order to determine the magnitude of cross-reactivity of the preparations, the same subject is administered three different preparations, *L. mexicana*, *L. tropica*, and *L. guyanensis*, at a given concentration simultaneously. This study allows the direct comparison of the three preparations and reduces the number of subjects in the study. The study is a dose escalation study which tests three dose levels of the microfluidized lysate preparations. Three cohorts of 20 subjects per clinical group are used. The dose of each preparation escalates for each cohort. Intradermal injections of saline and a 1:100 dilution of the diluent are administered as controls concomitantly with the preparations. Initially, 20 healthy subjects (Cohort 1) are administered microfluidized lysate preparations having total protein concentrations of 5 μg (0.1 ml ID). If no clinically significant reaction is observed within two days after administration in Cohort 1, Cohort 2 is administered preparations having 15.0 μg total protein (0.1 ml ID). If no significant reaction is